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Award Number: W81XWH-09-1-0386

TITLE: Synthetic Nanovaccines Against Respiratory Pathogens  
(SYNARP)

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REPORT DATE: July 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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<b>REPORT DOCUMENTATION PAGE</b>				<i>Form Approved</i> <b>OMB No. 0704-0188</b>	
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<b>1. REPORT DATE (DD-MM-YYYY)</b> 07/31/2010		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED (From - To)</b> 01 JUL 2009 - 30 JUN 2010	
<b>4. TITLE AND SUBTITLE</b>  Synthetic Nanovaccines Against Respiratory Pathogens (SYNARP)				<b>5a. CONTRACT NUMBER</b>	
				<b>5b. GRANT NUMBER</b> W81XWH-09-1-0386	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Bronich, Tatiana  Email: tbronich@unmc.edu				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  University of Nebraska Medical Center, Sponsored Programs Administration  987835 Nebraska Medical Center Omaha, NE 68198-7835				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  USA Med Research ACQ Activity 820 Chandler St. Fort Derrick, MD				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>  DOD	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b>  Approved for public release; distribution unlimited.					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> The overall goal of this proposal is to develop fully synthetic vaccines against respiratory infections using novel nanotechnology platforms based on safe and degradable adjuvant polymer systems that enhance antigen presentation and stimulate immunity. The proposal focuses on achieving the following specific technical objectives: 1) Develop molecular methods for intervention strategies employing novel synthetic nanovaccine platforms encapsulating DNA and protein/peptide antigens that elicit immune response against influenza H5N1; 2) Test the efficacy of nanovaccines-based intervention regimens against influenza H5N1 in animal models. 3) Adopt intervention strategies using most promising nanovaccine platforms to other respiratory infections, such as pulmonary F. tularemia, and test the nanovaccines efficacy in animal models (unfunded option). Focusing on respiratory infections, the leading cause of outpatient illness and a major cause of infectious disease hospitalization in U.S. military personnel, has the potential to develop effective, safe and affordable synthetic vaccines.					
<b>15. SUBJECT TERMS</b> Synthetic vaccines against respiratory pathogens					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>  UU	<b>18. NUMBER OF PAGES</b>  40	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U			<b>19b. TELEPHONE NUMBER (include area code)</b>

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## INTRODUCTION

The overall goal of this proposal is to develop fully synthetic vaccines against respiratory infections using novel nanotechnology platforms based on safe and degradable adjuvant polymer systems that enhance antigen presentation and stimulate immunity. Three novel versatile technology platforms will be evaluated: a) biodegradable polyanhydride nanospheres (BPN) carrying a polypeptide/protein antigen (Platform A); b) polymeric micelles of Pluronic block copolymer as DNA vaccine adjuvant (Platform B); and c) block ionomer complexes (BIC) for targeted delivery of DNA (or protein) antigen to the antigen presenting cells (APCs) (Platform C). The central hypothesis is that these polymeric nanoscale delivery systems can provide versatile platforms for development of effective, safe, and cost-efficient vaccines. The materials used in these platforms demonstrated immunomodulatory capabilities and can be targeted to specific populations of immune cells to elicit most efficient immune response. The best technology or their combination will be determined for future development.

The proposal is focusing on achieving the following specific technical objectives: 1) Develop molecular methods for intervention strategies employing novel synthetic nanovaccine platforms encapsulating DNA and protein/peptide antigens that elicit immune response against influenza H5N1; 2) Test the efficacy of nanovaccines-based intervention regimens against influenza H5N1 in animal models. 3) Adopt intervention strategies using most promising nanovaccine platforms to other respiratory infections, such as pulmonary *F. tularemia*, and test the nanovaccines efficacy in animal models (unfunded option).

The following milestones were set for July 1, 2009 – June 30, 2010:

1	<ul style="list-style-type: none"> <li>Assemble project teams at both sites of project performance (UNMC: PI's Kabanov, Bronich, and Hinrichs; ISU: Narasimhan, Mallapragada, and Wannemuehler) and identify project personnel to work on the research platforms</li> </ul>	Platforms A, B, and C	July 1, 2009 – August 31, 2009
2	<ul style="list-style-type: none"> <li>New essential equipment will be acquired</li> </ul>		July 1, 2009 – Dec 31, 2009
3	<ul style="list-style-type: none"> <li>IACUC will submitted and approved at both site of project performance</li> </ul>		
4	<ul style="list-style-type: none"> <li>IBC protocol will submitted and approved at UNMC</li> </ul>		
5	<ul style="list-style-type: none"> <li>Plasmid DNA containing H5 antigen will be expressed</li> </ul>	Platform A	
6	<ul style="list-style-type: none"> <li>Establish the procedure for the synthesis of the cationic copolymers based on the polypeptides</li> </ul>	Platform C	Sept 1, 2009 – March, 2010
7	<ul style="list-style-type: none"> <li>DNA plasmids encoding H5N1 HA and NA antigens will be constructed and expressed</li> </ul>	Platforms B and C	
8	<ul style="list-style-type: none"> <li>Synthesis of biodegradable polymers based on polyanhydrides will be carried out. The materials will be characterized for their morphology and thermal properties.</li> </ul>	Platform A	Jan 1, 2010 – June 30, 2010
9	<ul style="list-style-type: none"> <li>Cationic copolymers based on polylysine containing targeting groups will be synthesized and characterized</li> </ul>	Platform C	
10	<ul style="list-style-type: none"> <li>DNA plasmids encoding reporter gene for luciferase (Luc) in place of influenza proteins will be constructed and expressed</li> </ul>	Platforms B and C	
11	<ul style="list-style-type: none"> <li>Library synthesis methods for polyanhydride nanospheres will be validated</li> </ul>	Platform A	
12	<ul style="list-style-type: none"> <li>Production and purification of recombinant H5N1 protein will be carried out</li> </ul>	Platform A and C	

## BODY OF REPORT

### *Milestone 1*

The complex problem described in the grant cannot be addressed by any individual investigator and clearly requires a collaboration that crosses the boundaries of microbiology, biochemistry, virology, immunology, materials science, and engineering. Thus, we have assembled a team that consists of researchers from two institutions: University of Nebraska Medical Center (UNMC) and Iowa State University (ISU). The research team was assembled under the leadership of Dr.

Alexander Kabanov, Parke-Davis Professor of Pharmaceutical Sciences and Director of the Center for Drug Delivery and Nanomedicine, College of Pharmacy at UNMC. The senior investigators participated in the research project are: Dr. Surya K. Mallapragada, (Project Leader at ISU), Professor of Chemical and Biological Engineering at ISU and Program Director of Materials Chemistry and Biomolecular Materials at Ames Laboratory., an expert in the areas of biomaterials for drug, vaccine and gene delivery, and for tissue engineering; Dr. Tatiana Bronich, Associate Professor of Pharmaceutical Sciences and Associate Director of the Center for Drug Delivery and Nanomedicine (UNMC), an accomplished polymer chemist with extensive experience in the areas of material science and nanoscale assembly, including the block ionomer complexes for drug and gene delivery; Dr. Steven Hinrichs, Stokes-Shackleford Professor of Pathology and Chair, Department of Pathology and Microbiology, and Director of the University of Nebraska Center for Biosecurity. He is a clinical virologist and microbiologist who is leading the aerosol research program at UNMC; Dr. Balaji Narasimhan, Professor of Chemical and Biological Engineering at ISU, with extensive expertise in the areas of molecular design of biomaterials, controlled delivery of drugs, proteins, and vaccines, and combinatorial materials design; Dr. Michael J. Wannemuehler, Professor and Interim Chair in the Department of Veterinary Microbiology and Preventive Medicine (ISU), an expert in using animal models to evaluate immune responses (systemic and mucosal) following vaccination and/or infection; Dr. Cathy L. Miller, PhD., Assistant Professor in the Department of Veterinary Microbiology and Preventive Medicine (ISU), who has an expertise in reverse genetics that are essential for studies involving RNA viruses such as influenza; Dr. Peter Iwen, an Associate Professor in the Department of Pathology and Microbiology (UNMC), Biosafety officer and specialist in microbiology; Anthony R. Sambol, an Associate Professor in the Department of Pathology and Microbiology (UNMC), virology specialist and expert in aerosol vaccination techniques.

The personnel that is working on this project have been identified. At UNMC postdoctoral associate, Dr. Shaheen Ahmed, was recruited for this program. She is involved in design of plasmid DNA, animal vaccinations, and immunological studies of polymeric vaccines. At ISU Dr. Amanda Ramer-Tait is a research associate who will work part-time on this project and she will be supervised by Dr. Wannemuehler. Two graduate students have been hired to work on this project. Mr. Lucas Huntimer, who is an immunologist by training, has started

working on the protein/antigen side of the project and he is being jointly supervised by Drs. Wannemuehler and Narasimhan. Ms. Kathleen Ross, who is a biomedical engineer by training, has started working on the biodegradable polyanhydride nanoparticles side of the project and she is being jointly supervised by Drs. Narasimhan and Mallapragada. In addition, a graduate student, Bingqi Zhang, a chemical engineer, supervised by Mallapragada, recently started working on the project on the block copolymer platform to interface with the UNMC studies. All the project personnel have completed required training and are approved by Iowa State University to carry out the proposed studies.

#### *Milestone 2*

During the twelve month of the program the following equipment was acquired: two Biological Safety Cabinets and a dual chamber professional CO<sub>2</sub> cell culture incubator were purchased, installed and being put to use. This equipment is specifically designated for this project. Optima L-90K Preparative Ultracentrifuge (Beckman) was purchased, and installed. The personnel involved in the project (Caroline Roques, Zagit Gaymalov, Shaheen Ahmed) were trained by the company representative. Addition to these, Agilent 1200 Series LC HPLC system has been purchased, installed, and is in operational conditions. Kodak In-Vivo FX Multispec imaging system with PC from Carestream Health also purchased, and installed. The imaging system was also equipped with a small animal anesthesia system EZ-7000. The company representative trained the personnel involved in the project (Caroline Roques, Zagit Gaymalov, Shaheen Ahmed). Spectrophotometer Nanodrop 2000 and Nikon 90I Microscope and accessories have being acquired.

#### *Milestones 3 and 4*

Protocol of the animal research entitled “Synthetic Nanovaccines Against Respiratory Pathogens (SYNARP)” and corresponding IBC protocols (Protocol # 09-091-03) were reviewed and approved by UNMC Institutional Animal Care and Use Committee (IACUC). Both protocols were approved by Animal Care and Use Review Office, USAMRMC-MCMR-RP on April, 06, 2010. IBC protocols were submitted and approved (#10-04-007ABL2) at UNMC. The ISU team submitted an animal use protocol form to the ISU IACUC, which has been approved (Log # 1-10-6859-M) on February 4, 2010. In addition, these forms were submitted to DOD-ACURO for approval on June 15, 2010. Over the past month, there have been several e-mail messages exchanged with the ACURO protocol review specialist, Blair Warfield. To the best of

our knowledge, we have addressed all of their initial queries and are awaiting final approval from the ACURO in order to initiate the animal studies.

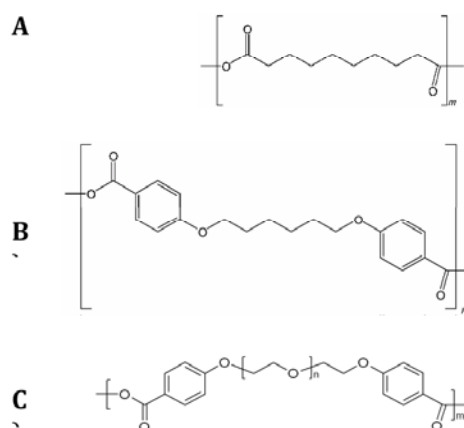
According to *Milestone 5* Huntimer and Wannemuehler cloned the full length H5 hemagglutinin (HA0) gene of strain A/Whooper Swan/Mongolia/244/05 into a pMT/BiP/V5-His B (Invitrogen, Carlsbad, CA) on expression vector designed for expression in *Drosophila melanogaster* Schneider 2 (S2) cells (Invitrogen) via polymerase chain reaction overlap extension techniques from a pHW2000 plasmid containing the H5 gene obtained from the Department of Infectious Diseases, St. Jude Children's Research Hospital. Full length sequence information for the H5 gene from the St. Jude H5N1 influenza seed virus 163243 is available at [http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=nucleotide&dopt=GenBank&RID=SDBJSRG001S&log\\$=nuclalign&blast\\_rank=1&list\\_uids=108671042](http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=nucleotide&dopt=GenBank&RID=SDBJSRG001S&log$=nuclalign&blast_rank=1&list_uids=108671042) (Accession DQ659326). The PCR overlap extension also allowed for reversion of the hemagglutinin cleavage site to wild type sequence so a full length HA0 protein will be expressed to maintain wildtype pre-cleavage structure. The pMT/BiP/V5-His B vector contains the metallothionein promoter and the BiP secretion sequence which when induced with copper sulfate in a stable transfected S2 cell the protein will be secreted into the cell media. Full length sequencing of the pMTH5 plasmid was performed and H5 gene sequence homology was obtained in comparison with the reference sequence provided on the NCBI GenBank database: DQ659326.1. *E. coli* One Shot TOP10 competent cells (Invitrogen) were used as a primary host for construction and propagation of the plasmids.

In support of *Milestone 8* Ross, Narasimhan, and Mallapragada have successfully synthesized and characterized biodegradable polyanhydrides based on sebacic acid (SA), 1,6-bis(p-carboxyphenoxy)hexane (CPH), and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG). **Figure 1** shows the structures of SA, CPH, and CPTEG.

#### Pre-polymer/Monomer Synthesis

SA: SA (40.0 g) (Aldrich Chemical, Milwaukee, WI) was refluxed in an excess of acetic anhydride (Fisher Scientific, Pittsburgh, PA) for 30 min. under nitrogen sweep. Acetic acid was removed by evaporating the solution at 50°C to dryness. The residue was purified by adding 40 ml chloroform (Fisher Scientific), followed by an addition of 400 mL of a 1:1 mixture of

anhydrous ethyl (Fisher Scientific) and petroleum ether (Aldrich Chemical). The solution was stirred for 10 min, filtered, and dried under vacuum overnight.



**Figure 1.** Repeat units of (A) poly(SA), (B) poly(CPH) and (C) poly(CPTEG). Here, m and n represent the number of repeating units of each monomer.

*CPH*: CPH was synthesized as described by Conix (1). The CPH diacid (40.0 g) was refluxed in an excess of acetic anhydride for 1 h under nitrogen sweep. Unreacted diacid was filtered by vacuum and the filtrate was evaporated to a volume of approximately 150 mL and refrigerated overnight. The resultant precipitate was washed three times with 100 mL aliquots of dry ethyl ether and dried under vacuum. The crude prepolymer was purified by dissolution in chloroform. Impurities were removed by filtration and the filtrate was evaporated to obtain purified CPH prepolymer.

*CPTEG*: To synthesize the CPTEG monomer, 45 mL of triethylene glycol, 100 mL of toluene, 300 mL of dimethyl formamide, and 0.897 mol of potassium carbonate were mixed in a round-bottom flask placed in an oil bath at 170°C (2). The addition of toluene allowed the azeotropic distillation of water from the reaction mixture prior to the reaction. Next, 0.684 mol of 4-p-fluorobenzonitrile was added and allowed to react overnight at 150°C. After cooling, all the solvents were removed using a rotary evaporator. The resulting dinitrile solution was hydrolyzed with a mixture containing equal volumes (50 mL) of water and acetic and sulfuric acid. The reaction was carried out at 160°C under a nitrogen atmosphere. The resulting diacid was precipitated using 1 L of deionized water. A white powder was obtained after successive washes with acetonitrile.

### Polymer Synthesis

For the CPH:SA system, Molar compositions 0:100, 20:80, 50:50, and 80:20 CPH:SA were synthesized by melt polycondensation of acetylated prepolymers at 180°C under high vacuum for 90 min (3). Crude polymer was dissolved in methylene chloride (Fisher Scientific) and precipitated in dry petroleum ether (Aldrich Chemical). The purified polymer was dried under vacuum overnight.

For the CPTEG:CPH system, 2 g of the monomer and 100 mL of acetic anhydride were added to a round-bottom flask and reacted for 30 min at 125°C. The acetic anhydride was removed in the rotary evaporator, and the resulting viscous liquid was polymerized in an oil bath at 140°C, under vacuum (<0.3 torr) for 90 min (2). The polymer was isolated by precipitating from methylene chloride into petroleum ether in a 1:10 ratio. The copolymer compositions synthesized were 20:80, 50:50, and 80:20 CPTEG:CPH.

#### Polymer Characterization

*Polymer Structure and Purity:* The structure and purity of the pre-polymers and polymers was verified using  $^1\text{H}$  NMR spectra obtained from a Varian VXR-300 MHz NMR spectrometer (Varian, Palo Alto, CA) and infrared spectra obtained from a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, Madison, WI).

*Molecular Weight and Molecular Weight Distribution:* CPH:SA copolymer molecular weights were determined using gel permeation chromatography (Perkin-Elmer) by eluting a solution of the polymer in methylene chloride in chloroform (Fisher) on a Waters 410 Differential Refractometer and 510 HPLC pump at a rate of 1.0 ml/min. In the case of the CPTEG:CPH system, the number average molecular weights were estimated by end group analysis from  $^1\text{H}$  NMR spectra.

*Thermal Properties and Morphological Characterization:* Thermal properties of the CPH:SA copolymers (glass transition temperature,  $T_g$ , and melting point,  $T_m$ ) were measured using a modulated differential scanning calorimeter (Modulated DSC 2910, TA Instruments, New Castle, DE). A temperature ramp of 10°C/min with a modulation of  $\pm 1^\circ\text{C}/\text{min}$ . was used. For the CPTEG:CPH system, the samples were heated in two cycles from -20 to 110°C at a rate of 5°C/min.

The structure and purity of the CPH:SA and CPTEG:CPH polymers synthesized were verified with NMR and IR spectroscopy. Peaks identifying the characteristics of the various copolymers (e.g., anhydride linkages, aliphatic and/or aromatic molecules, etc.) were identified and

confirmed with the spectroscopy experiments and no traces of solvents were observed (data not shown).

The homopolymer, poly(SA) and copolymers, 20:80, 50:50, and 80:20 CPH:SA were synthesized yielding weight-average molecular weights ranging from 20,000 to 40,000 and polydispersities between 2 and 4 (Table 1). In the case of the CPTEG:CPH system, the number average molecular weight of the polymers was estimated by calculating the degree of polymerization (DP) using the peak area normalized with the protons representing hydrolyzed and acetylated chains. The molecular weights of the CPTEG:CPH copolymers synthesized ranged from 4000 to 14,000 g/mol, which is in the sensitivity range of  $^1\text{H}$  NMR.

**Table 1.** Molecular Weights of Synthesized Polyanhydrides (CPH:SA using GPC and CPTEG:CPH using  $^1\text{H}$  NMR)

Copolymer composition	$M_n$	$M_w$	PDI
0:100 CPH:SA	18619	38038	2.04
20:80 CPH:SA	8841	26529	3.00
50:50 CPH:SA	5396	20608	3.82
80:20 CPH:SA	5515	20696	3.75
20:80 CPTEG:CPH	14124	-	-
50:50 CPTEG:CPH	8700	-	-
80:20 CPTEG:CPH	4700	-	-

**Table 2** shows the results of DSC analyses performed to measure the thermal properties ( $T_g$  and  $T_m$ ) of the CPH:SA copolymers. The CPH:SA family of polyanhydrides is semi-crystalline and melts at temperatures between from 79°C to 114°C. Table 2 also shows a summary of the thermal properties of the CPEG:CPH system. The CPTEG:CPH copolymers have  $T_g$ s below 20°C and hence are rubbery at room temperature. The rubbery state of these polymers at room temperature is desirable for processing into tablets. The DSC studies for the CPTEG:CPH copolymers did not exhibit any melting peaks, indicating that these polymers are amorphous.

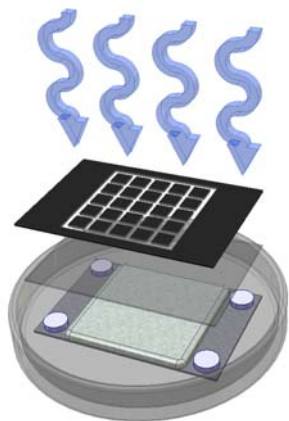
**Table 2.** Thermal Properties of Synthesized Polyanhydrides

Copolymer Composition	T <sub>m</sub> (°C)	T <sub>g</sub> (°C)
0:100 CPH:SA	79.0	62.2
20:80 CPH:SA	66.5	50.0
50:50 CPH:SA	50.2	-
80:20 CPH:SA	114.0	32.9
20:80 CPTEG:CPH	n/a	18
50:50 CPTEG:CPH	n/a	8
80:20 CPTEG:CPH	n/a	7

Library synthesis of BPNs based on CPH:SA and CPTEG:CPH has been successfully carried out (*Milestone 11*).

#### Multi-well Library Fabrication

Rapid prototyping was used to fabricate discrete solvent-resistant multi-well substrates



**Figure 2.** Schematic of photolithographic design of discrete thiolene-based multi-well substrates

capable of containing large enough fluidic volumes to support cell proliferation (**Figure 2**). This procedure is an adaptation of existing photolithographic techniques utilizing frontal-polymerizing optical resists (4). Major modifications were made to enable ease of use, speed, durability, reproducibility and cost. The method uses a thiolene-based, UV curing optical adhesive (NOA 81). Mechanistically, this involves the addition of a thiyl radical to a vinyl functional group, followed by radical transfer from the ensuing carbon radical to a thiol functional group. The extremely fast curing in the presence of oxygen using UV light, without the need for traditionally required photoinitiators means that fairly complex structures can be prototyped very easily and rapidly using a simple mask. In this work, the desired 5x5 array of discrete wells was

created using a photomask designed with a readily available drawing program and printed on a commercially available laser printer. The mask was then copied as many times as desired onto

transparencies with a photocopier. Four of the transparencies were overlaid (to ensure complete opacity) and affixed to a standard glass microscope slide with tape. Wells were given lateral dimensions of 5 x 5 mm, which when combined with a wall height of 2 mm produced space for liquid volumes of 200  $\mu$ L. To our knowledge, this represents a much greater well capacity than in any previous work.

Spacers were taped to regions of a second glass slide which fall outside the desired area of structure formation and served as an upper limit to feature height. The corners of the glass slides were removed with a glasscutter to allow them to fit within standard Petri dishes. Aluminum foil was cut into circles, flattened and laid into standard disposable Petri dish lids. The layer of aluminum served as a surface, which could be easily removed from the polymerized NOA 81. NOA 81 was then poured slowly onto the flat aluminum foil to a depth slightly less than the height of the spacers. The slide with spacers was then slowly laid down onto the NOA 81 layer, allowing capillary action to wet only the lower glass surface smoothly and with minimal bubble formation. The stack was then exposed to a collimated long-wave UV source at an intensity of 10 mW/cm<sup>2</sup> for 7 min. In order to provide increased resistance to the chlorinated solvents, high temperature, and high vacuum the substrate must endure during polyanhydride synthesis, it was necessary to polymerize a thin layer of NOA 81 in the bottom of each well. This was achieved by removing the photomask and exposing the naked substrate to 10 mW/cm<sup>2</sup> intensity UV light for 5 seconds. After precure, the aluminum foil layer was carefully peeled away from the structure and any unreacted NOA 81 remaining in the wells was removed with a blast of compressed air. Liberal amounts of ethanol from a spray bottle helped to more sharply define the features. After the ethanol evaporated, the substrates were postcured under UV light for 17 min at 10 mW/cm<sup>2</sup>. Finally, the substrates were thermally cured at 80°C for 12 h.

#### Prepolymer Solution Deposition

Libraries of varying concentration or mole fraction of CP:SA and CPTEG:CPH copolymers were rapidly deposited using robotics. Two programmable syringe pumps (New Era Pump Systems, Farmingdale, NY) in conjunction with three programmable motorized stages arranged orthogonally (Zaber Technologies, Richmond, British Columbia, Canada) served to fully automate depositions. The pumps and syringes were controlled by third-party macro software operating on the actuators' respective consoles. Complete 5 x 5 depositions were routinely completed in ca. 5 minutes.

### Combinatorial Synthesis of Polyanhydride Libraries

CPH:SA and CPTEG:CPH copolymer films were synthesized in the discrete well substrate from their corresponding prepolymers. CPH and SA prepolymers were dissolved in chloroform and deposited into the wells in various molar ratios. CPTEG:CPH prepolymers were dissolved in acetic anhydride and deposited in the same manner. With both systems the libraries were placed in a vacuum oven preheated to the necessary temperature (180°C for CPH:SA and 140°C for CPTEG:CPH) and 0.3 torr vacuum for the polycondensation reaction to occur. After synthesis, the polymer libraries were stored in desiccators.

### Library Characterization

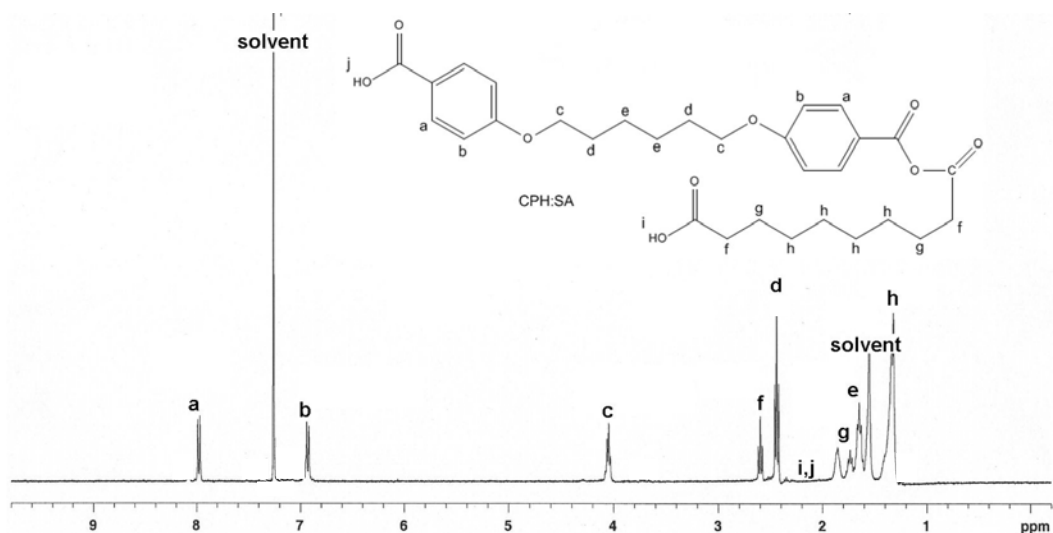
Copolymer structures were characterized by  $^1\text{H}$  NMR in deuterated chloroform on a Varian VXR 300 MHz spectrometer. Molecular weights were measured by GPC. GPC samples were dissolved in HPLC-grade chloroform and separated using PL Gel columns from Polymer Laboratories (Amherst, MA) on a Waters GPC system (Milford, MA). 50  $\mu\text{L}$  samples were eluted at 1 mL/min. Elution times were compared to monodisperse polystyrene standards (Fluka, Milwaukee, WI) and used to determine number averaged molecular weights ( $M_n$ ), and polydispersity indices. FTIR spectroscopy was conducted on a Nicolet Continuum infrared microscope (Thermo Scientific) in order to verify the ability of the robotics to accurately deposit linearly varying composition gradients. Two hundred scans were collected for each data point at a resolution of  $4\text{ cm}^{-1}$  and nitrogen purge flowrate of 30 SCFH.

### Nanoparticle Library Fabrication

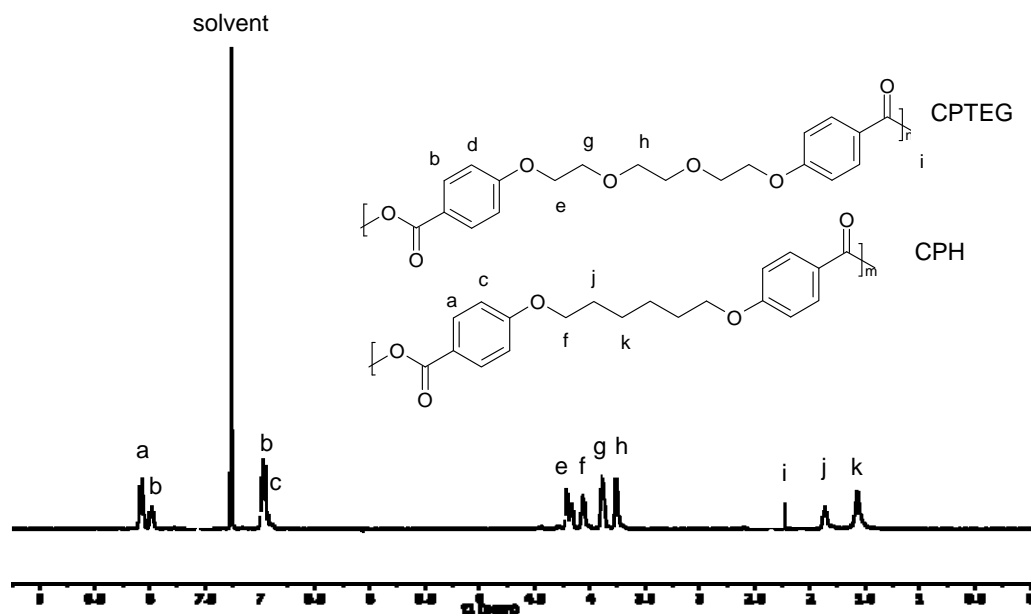
An automated nanoparticle fabrication apparatus was designed to create a multiplexed library of CPH:SA nanoparticles from the synthesized polymer film library. This process requires a modification to the film library deposition described previously (5). In the modified process, the thiolene based multi-well substrate was replaced by a multi-vial substrate to allow for larger holding volumes. The apparatus controlling the automated nanosphere fabrication process consisted of a series of 4 linear actuators (Zaber Technologies, Richmond, British Columbia, Canada) and 3 programmable syringe pumps (New Era Pump Systems, Farmingdale, NY) controlled by a third-party macro software operating on the actuators' respective consoles. This fully automated process was initiated by chloroform solution deposition into each vial of the multi-vial substrate, thus dissolving the polymer films with a resulting polymer concentration of ca. 25 mg/mL. Each solution was then homogenized for 60 s at 10,000 rpm to completely

dissolve the polymer in the chloroform. After that petroleum ether was dispensed into 15 mL tubes, each tube corresponding to a separate copolymer vial. Next, the polymer solution was withdrawn from a vial and dispensed into its corresponding tube of petroleum ether. During this time a nanoprecipitation process occurs in which the dissolved polymer is rapidly precipitated out in the presence of the non-solvent (petroleum ether) and the nanoparticles are formed. The optimal ratio of solvent to non-solvent was identified to be 1:40 for the fabrication of an average size of 300 nm polyanhydride particles. The nanoparticles were characterized with scanning electron microscopy (SEM) to analyze size and surface morphology using a JEOL 480A SEM (JEOL USA Inc., Peabody, MA).

NMR analysis was performed to ensure that the substrate did not interfere in the parallel synthesis of the copolymer libraries. Polyanhydride libraries with high concentrations were used to produce signals large enough to interpret. Representative spectra and lettered chemical shifts denoted in **Figure 3** for CPH:SA and **Figure 4** for CPTEG:CPH are marked as such by comparison to previous work (2-3). NMR spectra showed no difference between conventional syntheses methods of similar volumes performed in glass vials and the new combinatorial method performed in the multi-well substrates. Mole fraction gradients in the CPH:SA and CPTEG:CPH systems produced the expected changes in  $^1\text{H}$  NMR peak area. That is, peaks corresponding to the CPH portion of the copolymer (Peaks a-e in Figure 3) became less intense when compared to SA peaks (Peaks f-I in **Figure 3**) as the CPH content was reduced. Similar results were obtained with the CPTEG:CPH system (**Figure 4**). This suggests that the multi-well substrate did in fact isolate discrete copolymer compounds with minimal cross-contamination, and that the deposition robot created a smooth compositional gradient. It is also important to note that the end group peaks in these spectra are relatively small, suggesting that the syntheses did in fact drive off much of the prepolymeric acid groups, producing long chain polymers.



**Figure 3.** Proton NMR spectrum for combinatorially synthesized 50:50 CPH:SA conovlmer showing chemical shifts



**Figure 4.** Proton NMR spectrum for combinatorially synthesized 50:50 CPTEG:CPH copolymer showing chemical shifts

GPC was performed on various CPH:SA copolymers following synthesis in the wells and their molecular weights were compared to that of conventionally synthesized copolymers. As shown in **Table 3**, the copolymers had number-average molecular weights suitable for their processing into delivery devices such as tablets and nanoparticles, and the average polydispersity index was 2.2. Similar results were obtained with the CPTEG:CPH system. In this case, the molecular

weights were determined by end-group analysis using  $^1\text{H}$  NMR spectra. These values are consistent with previous work (2-3) on polyanhydrides synthesized by conventional polycondensation, and add further support to the success of the high throughput synthesis of polymers in the discrete wells.

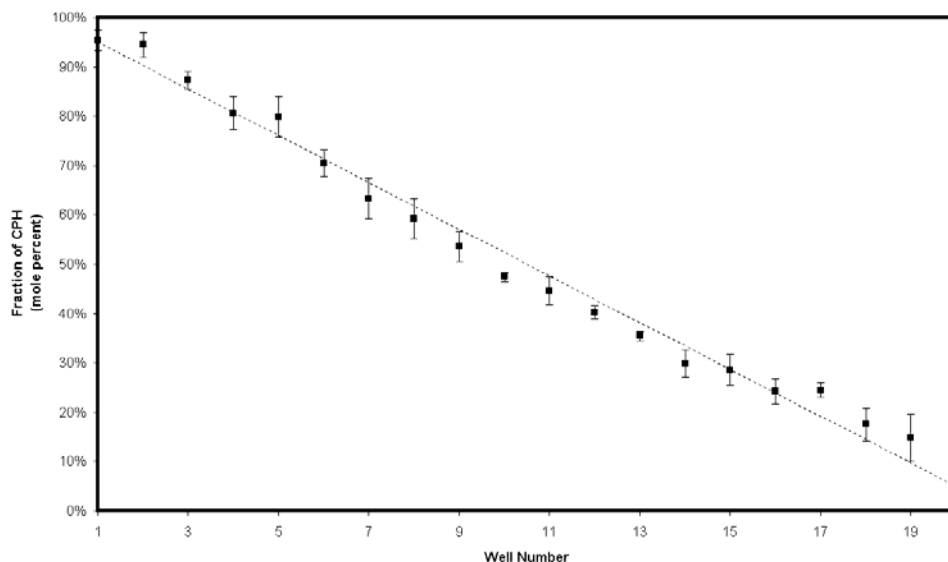
**Table 3.** Number average molecular weight and polydispersity index of combinatorially synthesized CPH:SA copolymers obtained by GPC

CPH:SA Copolymer	$M_n$ (Da)	Polydispersity index
0:100	11200	2.2
10:90	12700	2.5
20:80	9700	2.1
50:50	10800	2.4
80:20	13300	2.0
90:10	16500	2.0

The characteristic IR peak chosen for CPH is sharply defined at  $1605\text{ cm}^{-1}$  and represents the aromatic ring stretching of the CPH moiety. The feature chosen for poly(SA) is at  $1810\text{ cm}^{-1}$  and represents the carboxyl anti-symmetric stretching of the aliphatic-aliphatic anhydride bond.

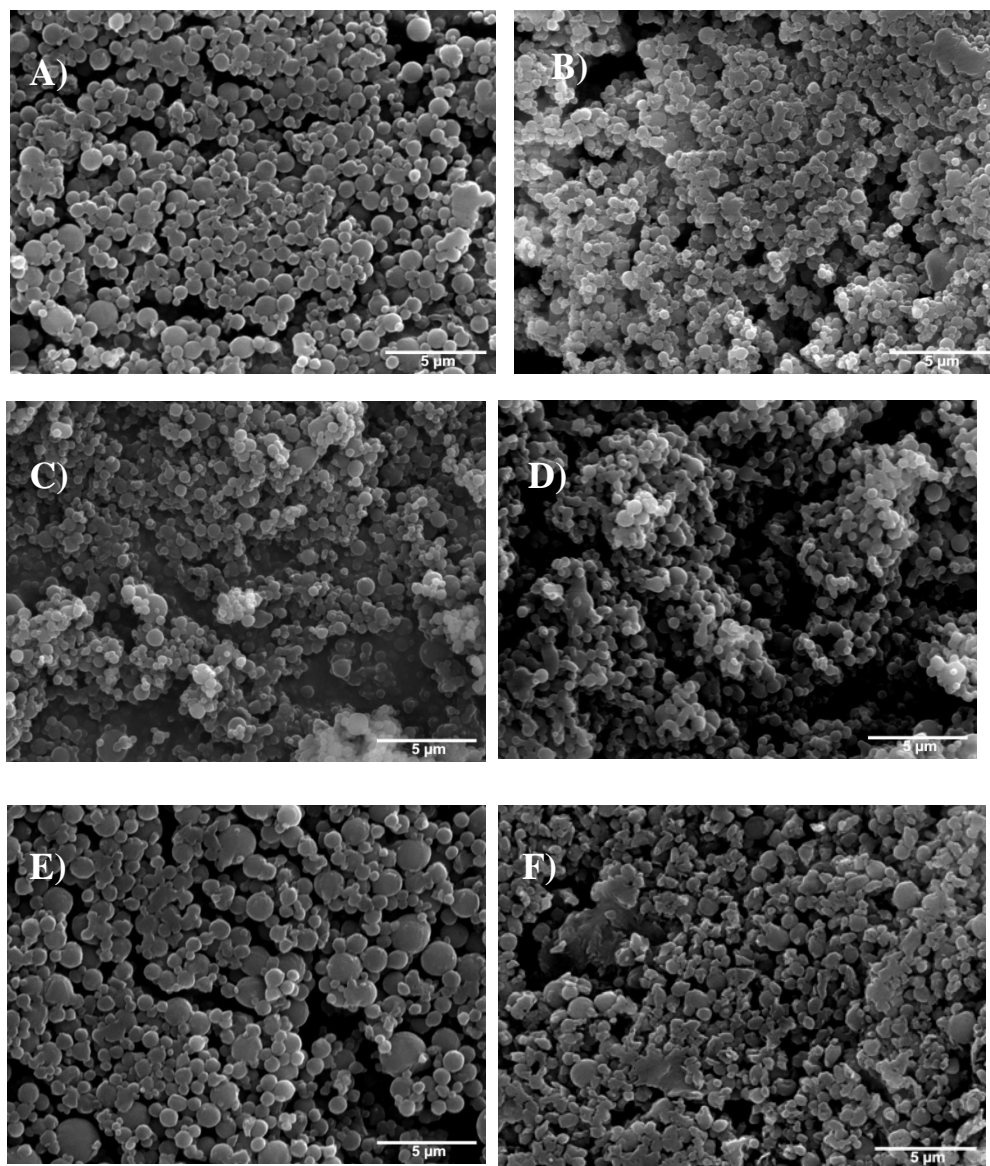
Each of the characteristic peaks was mathematically fitted with a standard normal distribution in order to calculate the area occupied by each band. The calculated peak areas for CPH ( $1605\text{ cm}^{-1}$ ) and SA ( $1810\text{ cm}^{-1}$ ) were divided by one another and further divided by their respective monomer molecular weights in order to account for the effect that differing values of  $M_r$  have on the area of the characteristic peak. The peak areas were also divided by their respective number of occurrences of characteristic peak functionality per monomer. Since the characteristic functionality for CPH (aromatic ring) and SA (aliphatic-aliphatic anhydride bond) both occur twice per monomer unit, the effect can be factored out. The measured area ratios were compared to a previously prepared calibration to elucidate the actual CPH mole fraction. **Figure 5** shows the resulting CPH:SA composition gradient as a function of position in the 5x4 array. The CPTEG:CPH libraries were characterized using NMR. The linearity of the profile spanning the library demonstrates not only the accuracy of the deposition apparatus but also the viability

of the thiolene/silicon substrate as a platform for high-throughput transmission FTIR characterization and parallel synthesis of polyanhydride libraries.



**Figure 5.** Comparison of predicted and experimentally measured composition in a 5x4 discrete library (95% confidence intervals)

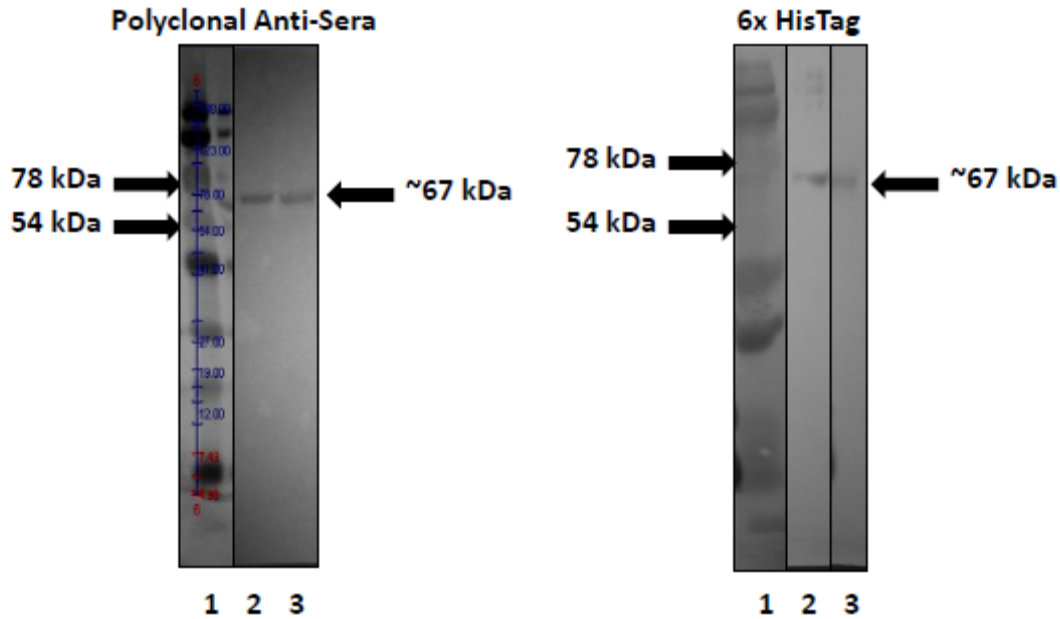
Following synthesis of the film libraries, nanoparticles of six copolymer compositions were rapidly fabricated using a nanoprecipitation technique. The multiplexed method enabled the simultaneous fabrication of nanoparticles ~100 times faster than conventional “one sample at a time” nanoprecipitation methods (6). Scanning electron photomicrographs of nanoparticles of six copolymer compositions are shown in **Figure 6**. It can be observed that the size, between 200 nm and 500 nm, and relative roughness are similar across polymer chemistries. These sizes and morphologies are in good agreement with those of conventionally synthesized nanoparticles (7).



**Figure 6.** SEM images of the discrete library of combinatorially fabricated nanoparticles starting with A) Poly(SA) B) 13:87 CPH:SA C) 25:75 CPH:SA D) 37:63 CPH:SA E) 50:50 CPH:SA and F) 63:37 CPH:SA. Scale bar: 5  $\mu\text{m}$ .

Huntimer and Wannemuehler are currently in the process of producing and purifying recombinant H5N1 protein (*Milestone 12*). The pMTH5 was co-transfected with pCoBlast, a plasmid DNA vector containing a blasticidin resistance gene allowing for successfully transfected cells, using Cellfectin II (Invitrogen) transfection reagent following protocols outlined in Santos et al (8). Cells were seeded in T-25 flasks with complete TNM-FH media containing 10% FBS and 0.5% Penicillin/Streptomycin and incubated at 28°C overnight. Co-transfections of pMTH5 and pCoBlast were performed at a ratio of 20:1. Plasmid DNA was

diluted in 500  $\mu$ L of serum-free TNM-FH media and 20  $\mu$ L of Cellfectin II was diluted separately in 500  $\mu$ L of serum-free TNM-FH media. The two dilutions were mixed at room temperature for 15 min. The 1 mL transfection solution was then added to cells after the media had been removed. The cells were incubated at room temperature for 5 h before 4 mL of complete TNM-FH selection media was added. After two days of incubation, selection media of complete TNM-FH containing 100  $\mu$ g/mL of blasticidin was used to replace the media. Fresh selection media was replaced every 72 h for two weeks for selection of blasticidin resistant cells. After two weeks of selection, cells were induced with 700  $\mu$ M of  $\text{CuSO}_4$  for 72 h. Cells were pelleted and lysed using protocols outlined from Invitrogen. Western blot assays were performed using polyclonal sera from pigs vaccinated with 1024 Hemagglutinin units of UV inactivated reverse genetics attenuated virus emulsified with emulsigen D adjuvant and HisDetector Nickel Conjugate (KPL, Gaithersburg, MD). Western blots using polyclonal pig sera and HisDetector Western Blot Kit (KPL) were performed after SDS-Page protein separation on 12% Mini-Protean TGX gels (Bio Rad, Hercules, CA) and transfer onto 0.2  $\mu$ m nitrocellulose membranes (Bio Rad). Polyclonal pig sera western blot membranes were blocked overnight at 4°C in 5% milk in Tris buffered saline with 0.05% Tween 20 (TBS-T). Membranes were washed with TBS-T and then sera diluted 1:200 in 1% milk TBS-T was added and incubated for two hours with gentle agitation at room temperature. Membranes were again washed and secondary anti-swine Ig alkaline phosphatase conjugated antibody diluted 1:1000 in 1% milk TBS-T was added and incubated for two hours with gentle agitation at room temperature. Membranes were washed and then developed using Fast Red TR/Naphthol As-Mx alkaline phosphatase substrate tablets (Sigma, St. Louis, MO). HisDetector western blots were performed according to manufacturer's protocols. Bands were detected via western blots and the recombinant H5 protein had an apparent molecular size of 67 kDa that is consistent with the HA0 translational size (**Figure 7**).



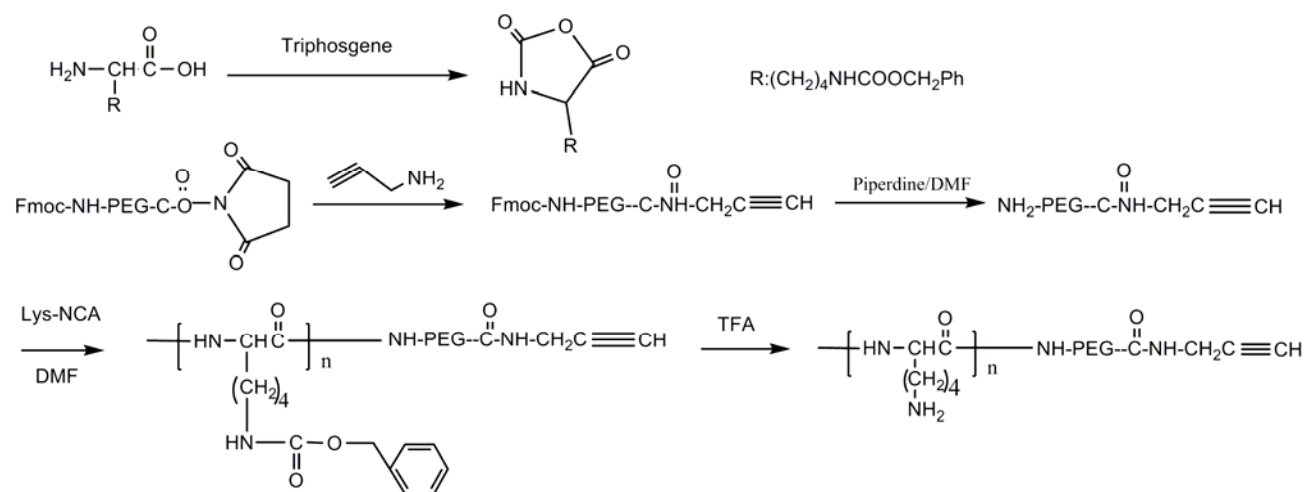
**Figure 7.** Western blots of *Drosophila* S2 cell lysate against polyclonal pig sera from animals vaccinated with inactivated H5N1 influenza virus (Polyclonal Anti-Sera) or against HisDetector Nickel Conjugate (6X HisTag). Lane 1 represents SDS-Page Lonza ProSieve molecular weight markers. Lanes 2 and 3 are two different S2 colony cell lysates post blasticidin selection.

S2 cells were scaled up for protein production seeding T-125 flasks at  $5 \times 10^6$  cells/mL in complete TNM-FH media. Cells were induced with  $\text{CuSO}_4$  as described earlier. Cells were pelleted and the media was used for isolating the HA0 protein using Profinity IMAC resin (Bio Rad) according to manufacturer protocols. SDS-Page, western blot, and sequencing analysis of proteins eluted from the column showed non-specific bovine serum albumin (BSA) from the growth media as well as the H5 protein of interest. Pretreatment with increasing concentrations of Imidazole to prevent non-specific binding of the BSA showed little improvement in protein purity. SF900-II SFM serum free media (Invitrogen) was obtained and stable transfected cell lines are being established using this base media to increase protein purity and prevent non-specific protein. Proteos, a protein production contract research organization in Kalamazoo, MI with expertise in the *Drosophila* S2 cell system as well as protein purification, has also been contracted for stable cell line transfection and purification of H5 HA0 protein and are currently in process. Purified HA1 of influenza A/ recombinant protein purified from *E. coli* was also obtained from Dr. Jerry Keith. It is widely considered that glycosylation of the HA protein of influenza via mammalian or insect cells contributes to proper conformational epitopes needed for

humoral immune protection from influenza infection however and bacteria lack the translational mechanisms of glycosylation.

According to the *Milestone 6* the synthesis of the cationic copolymers based on the polypeptides was performed. Specifically, one sample of cationic graft-copolymer based on polylysine (PLL-g-PEG) has been synthesized and characterized. The PLL-g-PEG graft copolymer was prepared by reacting succinimidyl derivative of poly(ethylene glycol) (PEG) propionic acid ( $M_w=5000$ , NOF Corporation) with PLL ( $M_n=12,000$ , Sigma) in phosphate buffer for 2 hrs at room temperature. The product was purified by exhaustive dialysis (SpectraPor membrane, molecular weight cutoff size 12-14 kDa) and characterized by  $^1\text{H}$  NMR spectroscopy using  $\text{D}_2\text{O}$  as a solvent on a Varian 500 MHz spectrometer. From  $^1\text{H}$  NMR spectra of the synthesized PLL-g-PEO, the numbers of PEO chains grafted onto a PLL backbone was calculated to be 1.55. Reverse titration was carried out to determine the concentration of amino group in PLL-g- PEO solution, which was determined to be 6 mM.

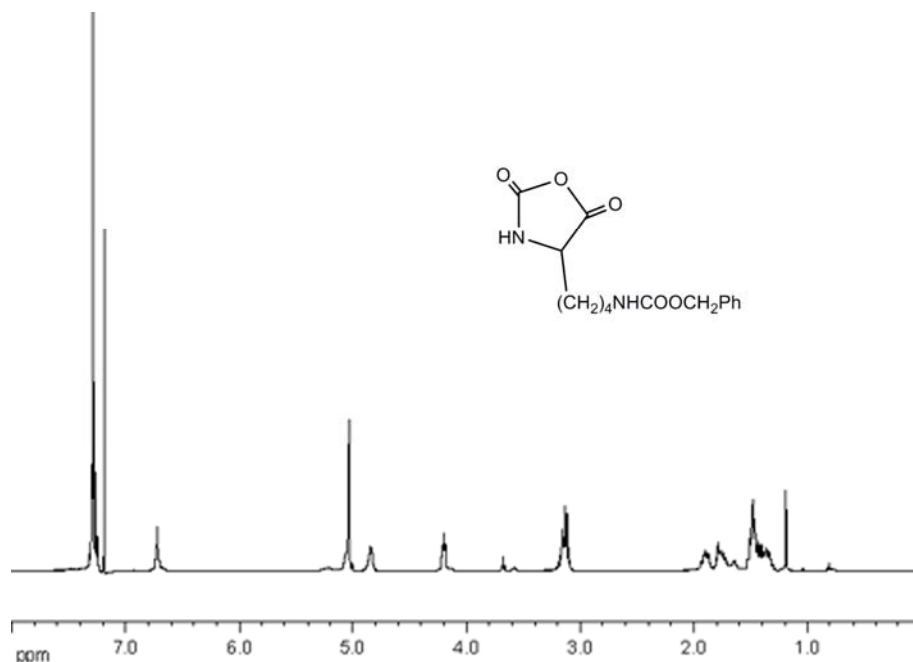
The step-by-step procedure for the synthesis of PEG-*block*-PLL copolymer containing terminal reactive groups was developed as presented in **Scheme 1**.



**Scheme 1.** Scheme of synthesis of alkyne-modified PEG-*b*-PLL copolymer

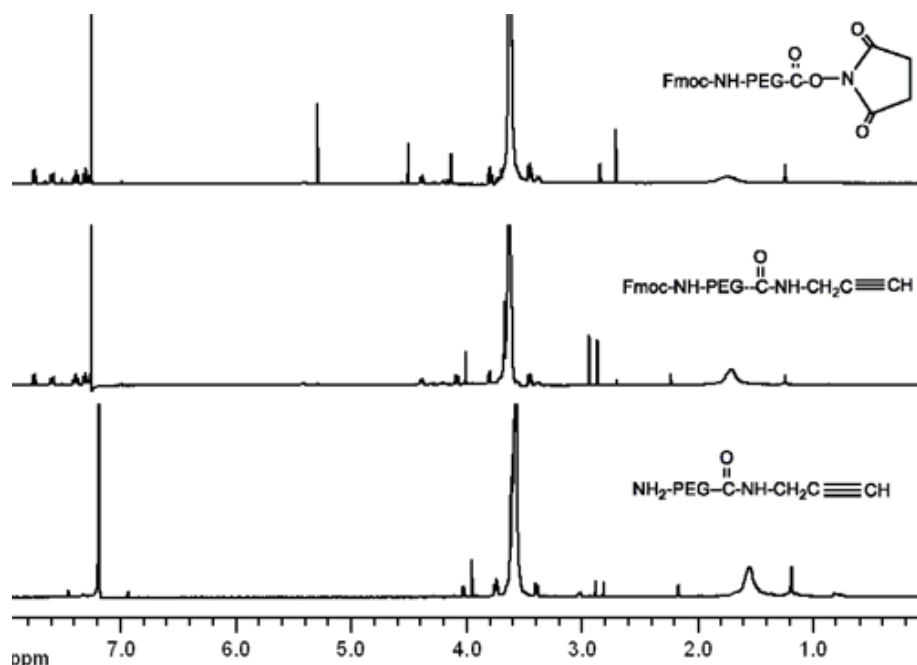
At first the monomer,  $\omega$ -carbobenzyloxyl-L-Lysine N-carboxylanhydride ( $\omega$ -carbobenzyloxyl-L-lysine- NCA) was synthesized by the treatment of the suspension of  $\omega$ -carbobenzyloxyl-L-lysine

in THF at 50°C until the clear solutions were formed. The resulting Lys-NCA was then recrystallized from THF/hexane mixture twice and its structure was confirmed by  $^1\text{H-NMR}$  (**Figure 8**).



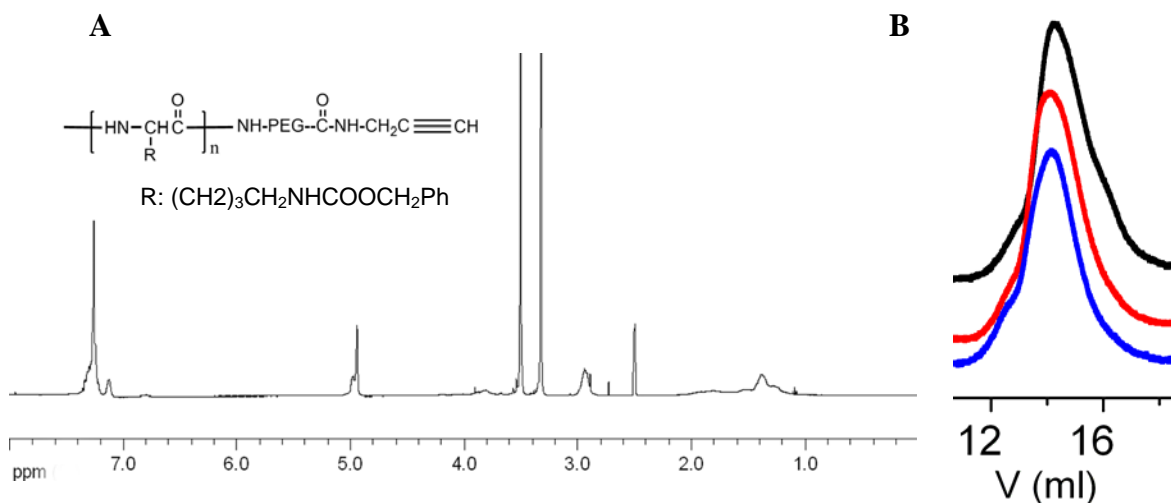
**Figure 8.**  $^1\text{H-NMR}$  spectrum of  $\omega$ -carbobenzoyloxyl-L-lysine- NCA. Solvent:  $\text{CDCl}_3$

The synthesis of amino-PEG-alkynyl was started from the commercial Fmoc-protected PEG- succinimidyl ester (M.w. 5,000, Creative PEGWork). It was allowed to react under dry conditions with propargyl amine to yield Fmoc-NH-PEG-propargyl which is subsequently was deprotected using piperidine/DMF mixture. The structures of the intermediate products and alkynyl-modified copolymer were confirmed by  $^1\text{H-NMR}$  (**Figure 9**).



**Figure 9.**  $^1\text{H}$ -NMR spectra of (i) Fmoc –protected PEG- succinimidyl ester, (ii) Fmoc-NH-PEG propargyl; and (iii) amino-PEG-alkynyl. Solvent:  $\text{CDCl}_3$

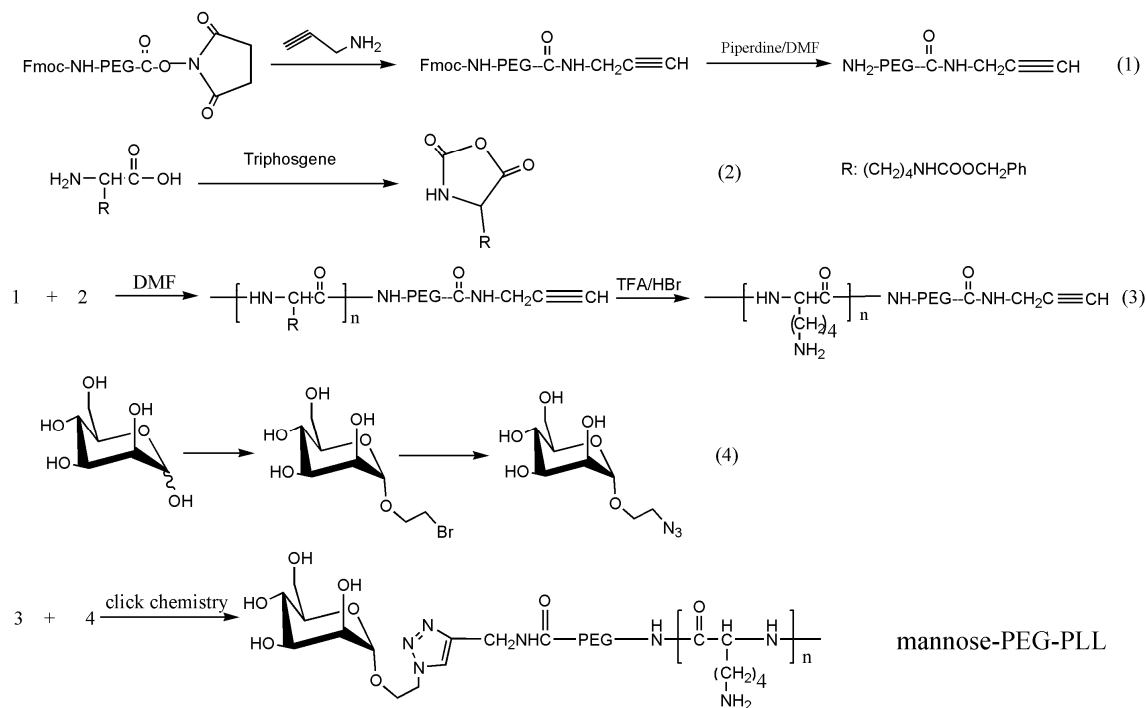
The product was then used directly as a macroinitiator in lysine-NCA polymerization performed in DMF, under oxygen-free condition at  $40^\circ\text{C}$ , reaction time was 72 hours. The structure of the prepared copolymers was confirmed by  $^1\text{H}$ -NMR. and gel permeation chromatograph( GPC) (**Figure 10**). The molecular weight and molecular weight distribution ( $\text{PDI} = \text{Mw}/\text{Mn}$ ) were measured by GPC (Viscotek) against narrow molecular weight distribution poly(methyl methacrylate) in DMF at a flow rate of  $1.0\text{ml}/\text{min}$  at  $45^\circ\text{C}$  with reflection index, laser light and viscosity detector. GPC profiles of the synthesized copolymers are presented in **Figure 10B**. Deprotection of  $\alpha$ -amino group of lysine residues group was carried in TFA using a 33% hydrobromic acid solution in galatic acetic acid. Typically, 8 ml of TFA was added to 0.8 g propargyl-PEG-b-PLL(R) to dissolve the polymer, a solution of 33% HBr in galatic acetic acid (4 times excess with respect to the R group) was added to the polymer solution, the mixture was stirred at room temperature for 1 hour. The reaction mixture was then poured into an excess of cold diethyl ether. The precipitate was dissolved in methanol, then precipitate with diethyl ether twice. Altogether, three samples of  $\text{PEG}(114)\text{-}b\text{-PLL}(x)$ , where  $x = 62, 150, 206$  have been synthesized and characterized .



**Figure 10.** (A)  $^1\text{H}$ -NMR spectra of alkyne-PEG-*b*-PLL(R) copolymer. Solvent: DMSO- $d_6$ . (B) GPC profiles of alkyl-PEG-*b*-PLL(R): PEG<sub>114</sub>-*b*-PLL<sub>62</sub>, PDI = 1.27 (black), PEG<sub>114</sub>-*b*-PLL<sub>150</sub>, PDI = 1.25 (red) and PEG<sub>114</sub>-*b*-PLL<sub>206</sub>, PDI = 1.19 (blue), respectively, Eluent: DMF (0.02 M LiBr); Flow rate: 1 ml/min.

#### Milestone 9

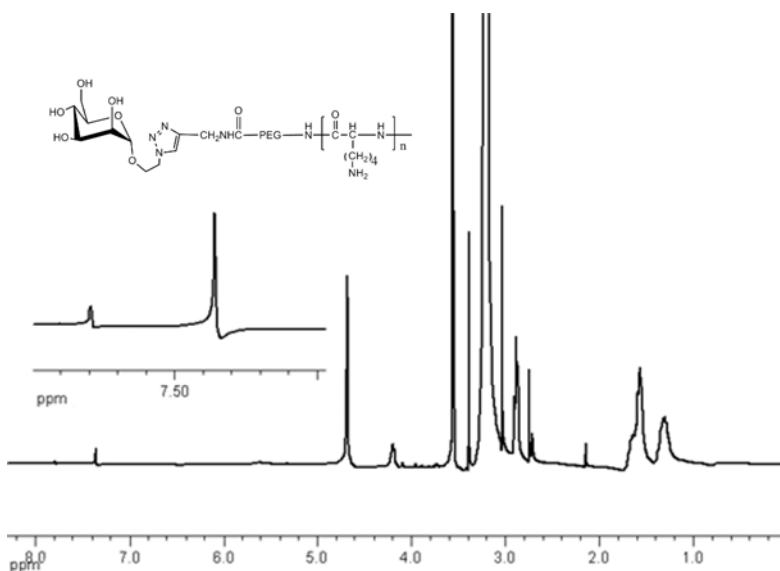
The following synthetic scheme was implemented for the synthesis of PEG-*block*-PLL copolymers containing mannose targeting groups:



**Scheme 2.** Step-by-step procedure for the synthesis of mannose-modified PEG-*b*-PLL copolymer

Mannose azide (**4**, **Scheme 2**) was synthesized using two-step procedure. First, 1.5 g of Amberlite IR-120H was suspended in 11.5ml 2-bromoethanol, and the mixture was heated to and retained at 90°C for 30min, then 1.5g D-mannose was added to the mixture. The reaction was allowed to proceed to 3 hours at 90°C, then the reaction mixture was filtrate to remove Amberlite IR-120H, washing the filtrated solid with further 2 ml of 2-bromoethanol. The excess of 2-bromoethanol was distilled off under reduced pressure, the resulting sticky residue was dissolved in methanol and 5g of SiO<sub>2</sub> was added. The solvent was removed under reduced pressure and the silica-supported reaction mixture was loaded onto a column previously filled with SiO<sub>2</sub> and pre-eluted with ethyl acetate / methanol 19:1 vol/vol. After elution with the same solvent mixture, appropriate fractions were collected and the solvents were removed under reduced pressure to give 2'-Bromoethyl- $\alpha$ -D-mannopyranoside as light yellow oil. At the next step, 0.91g of sodium azide and 2g of 2'-bromoethyl- $\alpha$ -D-mannopyranoside were dissolved in 5ml of water. Then 30ml of acetone was added and the resulting mixture was heated up to reflux (76°C) and stirred at this temperature for 20 h. Acetone was then removed under reduced pressure. The resulting oily residue was purified on silicagel column using ethyl acetate / methanol (19:1 vol/vol) mixture. Appropriate fractions were collected and the solvents were removed under reduced pressure to give (**4**) as light yellow syrup.

Equivalent amounts of alkyne-terminated PEG-*b*-PLL copolymer (**3**, **Scheme 2**) and mannose azide (**4**, **Scheme 2**) were dissolved in 1:1 mixture of water and methanol. The 5 mol% of copper(II) sulfate pentahydrate was added to the above solution, followed by 10 mol% sodium ascorbate. 5 mol% N,N,N',N'',N'''-Pentamethyldiethylene netriamine (PMDETA) was used as the ligand for the copper ions. The reaction was allowed to proceed 24 hours at room temperature, then the reaction mixtures were dialyzed against deionized water for 2 days and freeze-dried to give mannose-PEG<sub>114</sub>-*b*-PLL(x). The structure of the prepared copolymers was confirmed by <sup>1</sup>H-NMR (**Figure 11**).



**Figure 11.**  $^1\text{H}$ -NMR spectra of mannose-PEG<sub>114</sub>-b-PLL(x) copolymer. Solvent: D<sub>2</sub>O.

The characteristics of the synthesized mannose-PEG-b-PLL copolymers are presented in **Table 4**.

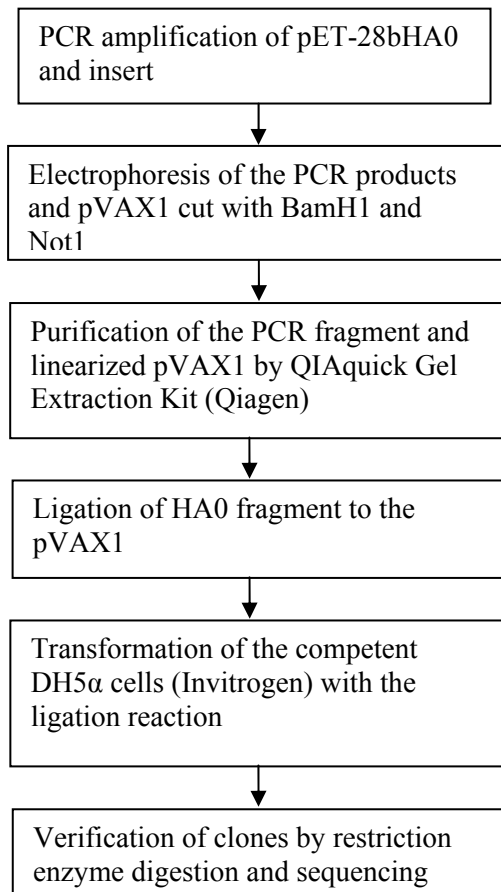
**Table 4.** Characteristics of mannose –PEG-b-PLL copolymers

Composition	PDI*	Mannose %
Mannose-PEG <sub>114</sub> -PLL <sub>62</sub>	1.27	87%
Mannose-PEG <sub>114</sub> -PLL <sub>150</sub>	1.25	72%
Mannose-PEG <sub>114</sub> -PLL <sub>206</sub>	1.19	95%

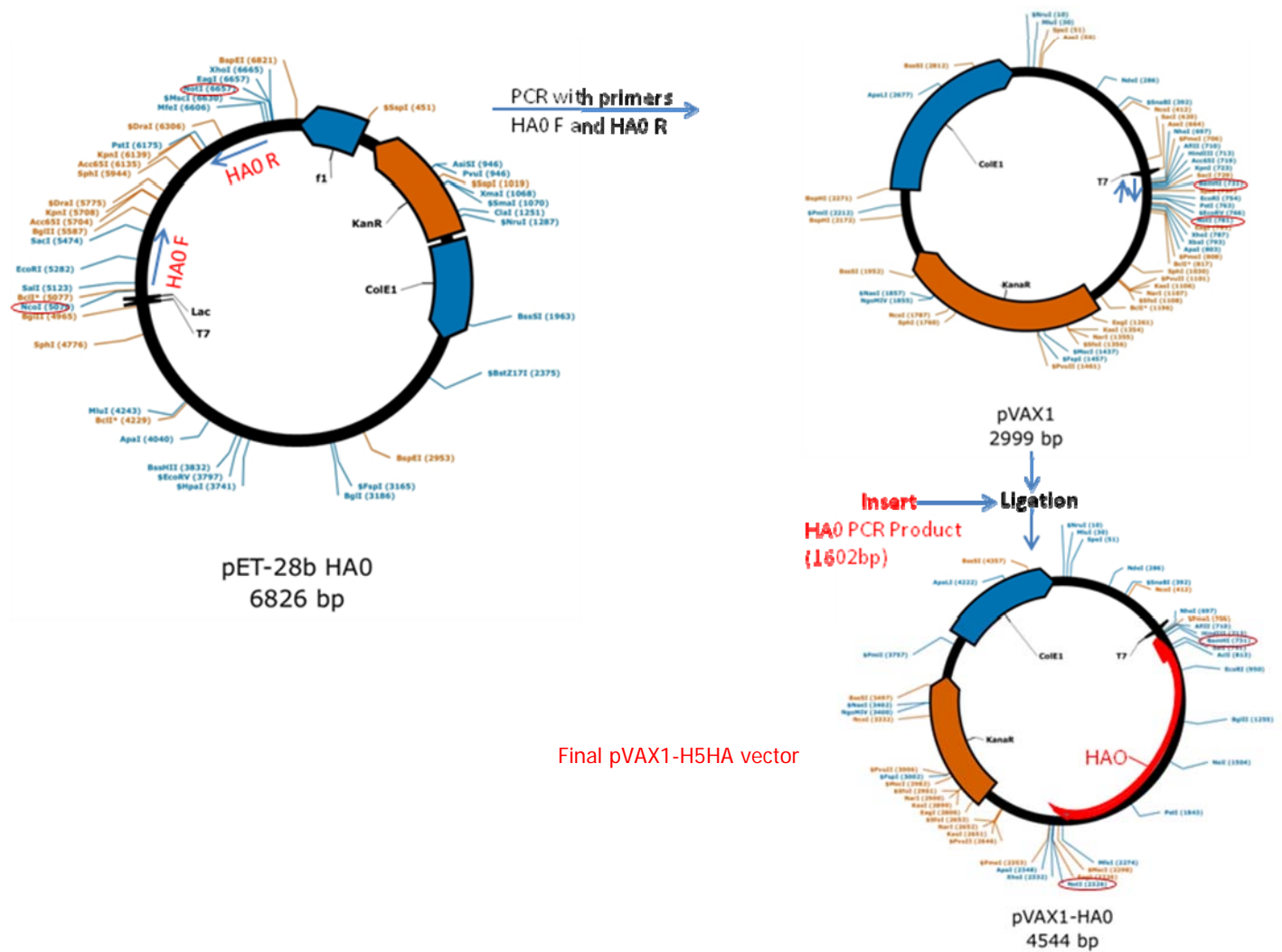
\* Polydispersity index, PDI, was determined by GPC

In support of the design of DNA plasmid encoding reporter gene in place of influenza proteins (*Milestone 10*), the DNA sequence coding for the Luciferase reporter system has been subcloned from the gWiz-Luc plasmid and inserted into the pVAX1 backbone. After completion of the subcloning step, the pVAX1-Luc plasmid generated has been introduced into competent DH5 $\alpha$  *E. Coli* bacteria, amplified and purified. Part of the purified plasmids has been used for the DNA sequencing of the insert. The functionality of the encoded protein has also been checked through an *in vitro* transfection assay. Briefly, HEK293T cells were transfected with the pVAX1-Luc plasmid combined to Lipofectamine 2000. Proteins were then extracted and Luciferase expression was quantified using a Luciferase assay kit.

The plasmid encoding the H5N1 HA (pET-28bHA0) was kindly provided by Dr. Jerry Keith, (NIH/NICHD) and subcloned into the pVAX1 backbone. The plasmid was then expanded in DH5 $\alpha$  *E. Coli* bacteria and purified. Purified pVAX1-H5HA plasmid has been sequenced and shown 100% sequence homology of the insert. The flowchart of the subcloning protocol is presented in **Figure 12**, and the vector construction diagram is depicted in **Figure 13**.



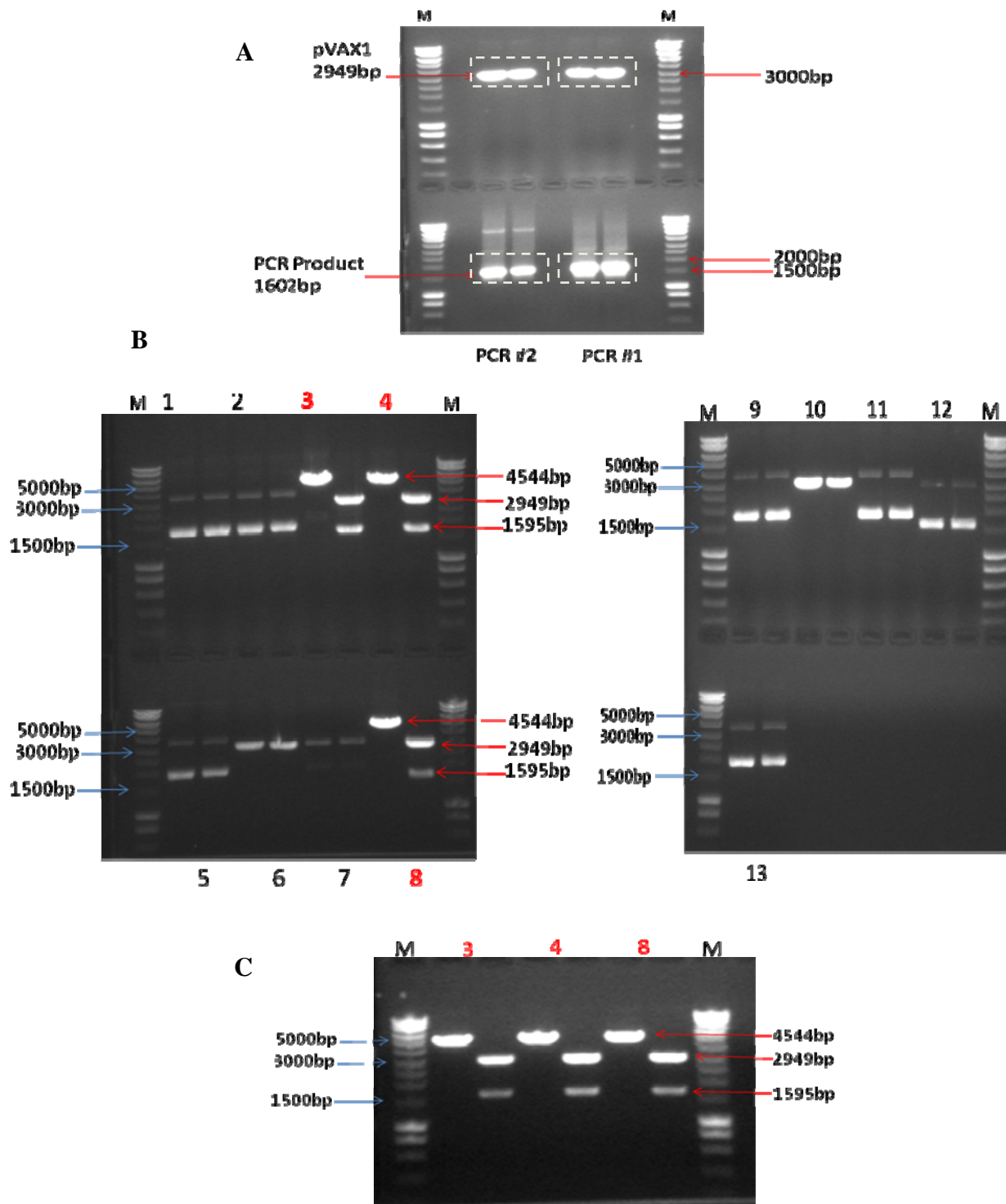
**Figure 12.** Flowchart of pVAX1-H5HA vector construction protocol.



**Figure 13.** pVAX1-H5HA vector construction diagram

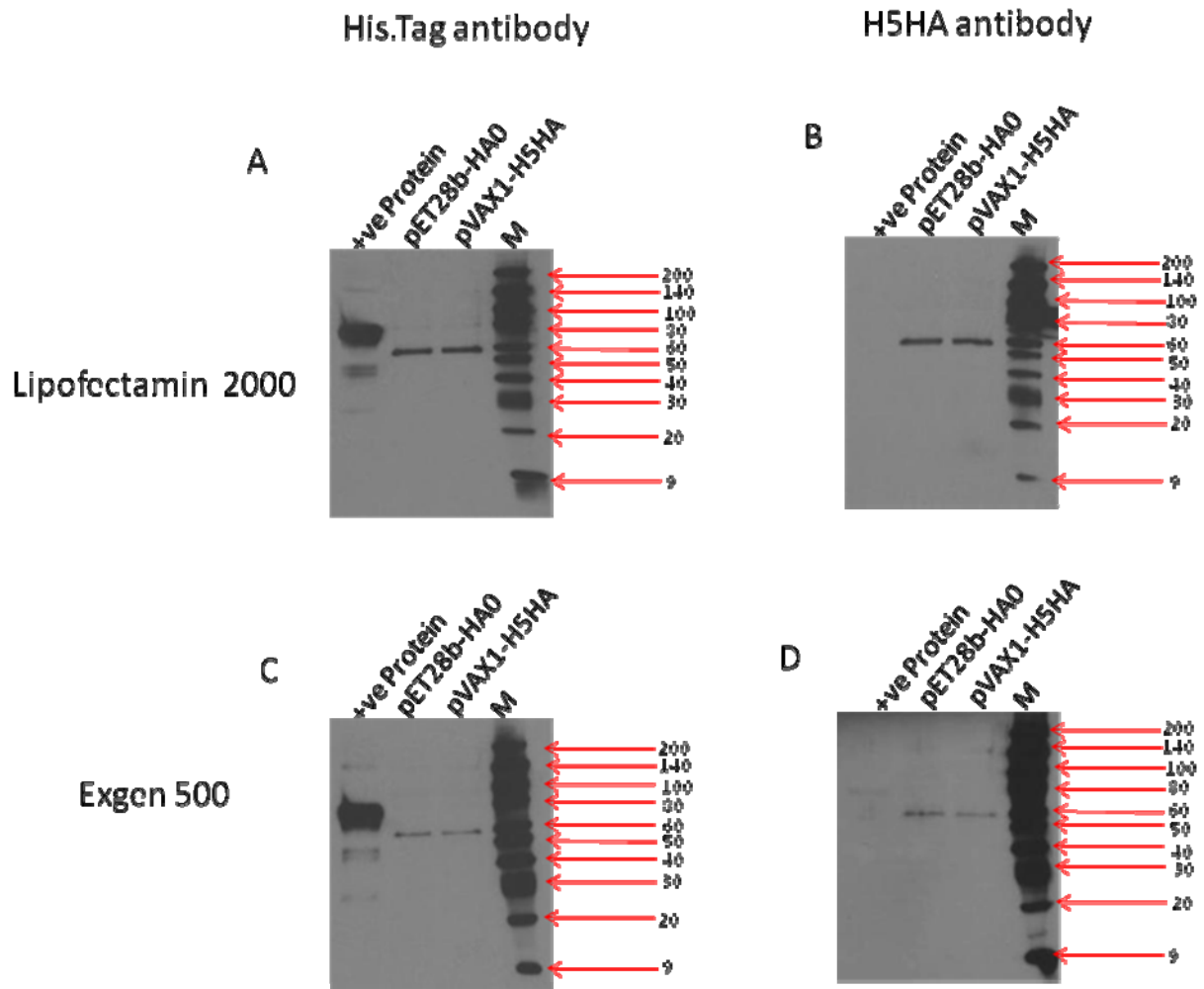
The pET28b-HA0 construct is designed to represent the mature configuration of HA0 of influenza virus A/Vietnam/1203/2004. In the pET28b expression vector, HA0 gene is cloned into the Nco1 site at the 5'-end of the gene and into the Not1 site at the 3'-end of the coding region. To facilitate cloning into the pVAX1 expression vector, BamH1 and Not1 restriction site incorporated into the construct. To incorporate these restriction sites, we designed the PCR primers for 5'- and 3'-end of the H5HA gene. The 5'-end primer was CTC GGA TCC GTC ATG GGT GAT CAG ATT TGC ATT and 3'-end primer was GCG GCC GCC TCA TTA ATG GTG ATG ATG ATG GTG. The amplified DNA fragment treated with BamH1 and Not1

restriction enzymes and purified by agarose gel electrophoresis (**Figure 14A**). pVAX1 vector was also treated with the same restriction enzymes and purified by agarose gel electrophoresis (**Figure 14B**). The BamH1/Not1 DNA fragment then was ligated into BamH1 and Not1 restriction sites of the pVAX1 expression vector. The ligated plasmid was transformed into *E. coli* DH5- $\alpha$  and selected on LB-agar plates in the presence of Kanamycin (50 $\mu$ g/mL). We received 13 antibiotic resistant bacterial colonies. Mini-preparations of all 13 clones were verified by restriction enzyme digestion and electrophoresis for their size (**Figure 14B**). But out of 13 clones, only 3 clones had as expected the right size (clone no. 3, 4 and 8) (**Figure 14B**). We also performed midi-preparations of these 3 plasmids with the insert, using Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA), and verified their size by restriction enzyme digestions (**Figure 14C**). Plasmids 3, 4 and 8 were further used for verification by sequencing. For DNA sequencing of HA0 insert, we used two vector-specific primers, T-7 promoter- specific sense primer, the BGH reverse primer, and internal HA0 gene specific primers (forward primer, 5'- ATC CTA ATG ATG CGG CAG AGC AGA-3' and reverse primer, 5'- TAT TTC TGA GCC CAG TCG CAA GGA-3'). Sequence analysis of those 3 plasmids confirmed that all of them carried the correct HA0 gene sequence. We assigned a new name, **pVAX1-H5HA**, for the prepared construct. The plasmid of clone 8 was chosen to be used in all the following experiments.



**Figure 14.** Agarose gel electrophoresis of different steps of pVAX1-H5HA vector construction. **A**, pVAX1 vector and PCR products were treated with BamH1 and Not1 restriction enzymes and purified ; **B**, mini-preps of plasmid 1-13 treated with BamH1 and Not1 restriction enzyme digestion; and **C**, midi-preps of plasmid 3, 4 and 8 treated with BamH1 and Not1 restriction enzyme digestion.

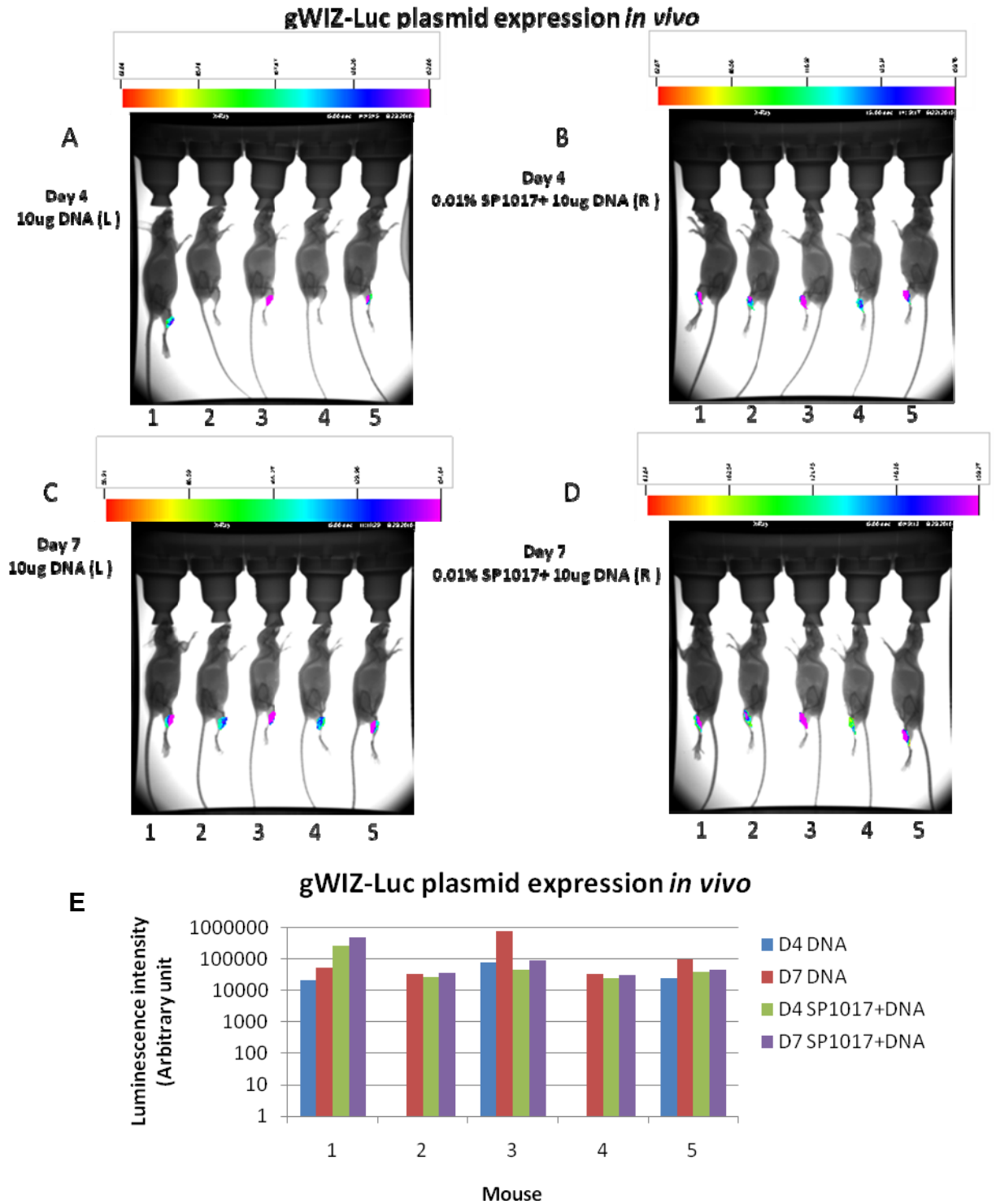
This constructed plasmid has been tested for its *in vitro* expression in HEK293T cells. HEK293T cells were grown in Dulbecco's modified Eagle's medium *containing 10% fetal bovine serum and 1% penicillin and streptomycin* in 6-well plates. At approximately 70-80% of confluency the cells were transfected with pVAX1-H5HA and pET28b-HA0 constructs with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or Exgen 500 (Fermentas, Glen Burnie, MD) according to the manufacturer's protocol. To check the protein expression, proteins were extracted 48 h after transfection. As carboxyl terminal portion of both H5HA and HA0 fragments contain six Histidine residues (6xHis), we purified the extracted proteins using MagneHis Protein Purification System according to manufacturer's protocol (Promega, Madison, WI) in the presence of protease inhibitors. 6xHis tagged H5 (H5N1) (A/Vietnam/1203/2004) protein (amino acid 18-530) (eEnzyme, Montgomery Village, MD) was used as a positive control. For Western blot analysis, purified proteins were resolved on a 4-20% Tris-HCl SDS gel and electroblotted to PVDF membranes. The membranes with blotted protein were blocked for 1 h with 1% Alkali-Soluble Casein in TBS, followed by 2 h incubation with mouse monoclonal anti-His.Tag antibody (Novagen, Gibbstown, NJ) and mouse monoclonal anti-H5 Hemagglutinin of A/Vietnam/1203/04 Influenza Virus (VN04-9) antibody (Rockland, Gilbertsville, PA) diluted 1:1000 in TBS-Tween at room temperature. Subsequently, the membranes were washed and incubated with rabbit anti-mouse IgG HRP (Sigma) diluted 1:30,000 in TBS-Tween for 1h. Following washes, visualization was performed with ECL-kit (Pierce, Rockford, IL). The electrophorethogram is presented in **Figure 15**.



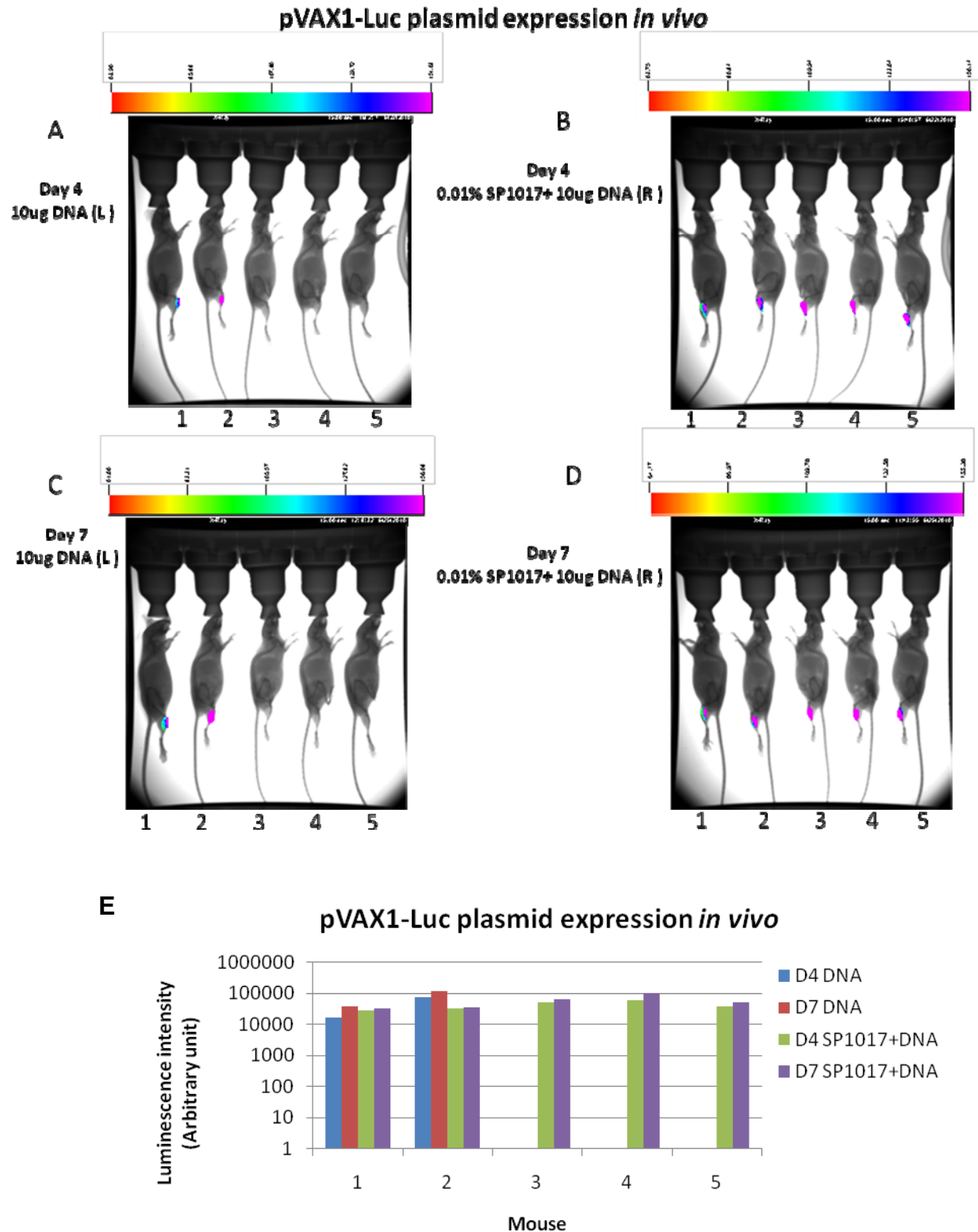
**Figure 15** .Expression of H5N1 HA in HEK 293T cells transfected with pVAX1-H5HA and pET28b-HA0 vectors. Transfections were carried out using the transfection reagents Lipofectamin 2000 (Panel A and B) and Exgen 500 (Panel C and D). Purified proteins were analyzed by Western Blot using mouse monoclonal anti-His.Tag antibody (Panel A and C ) and mouse monoclonal anti-H5 Hemagglutinin of A/Vietnam/1203/04 Influenza Virus (VN04-9) antibody (Panel B and D).

We further determined whether the pVAX1-H5HA and pVAX1-Luc plasmids (pDNA) will be expressed in tibialis anterior muscle of a mouse post *i.m.* administration. Female Balb/c mice (6-8 week-old) were used in these experiments. All experiments involving animals were conducted in accordance with national animal welfare guidelines and approved by the Institutional Animal Care and Use Committee at UNMC.

To this end, 10 µg of gWIZ-Luc pDNA (reference plasmid), pVAX1-Luc pDNA (control plasmid) and pVAX1-H5HA pDNA were administered into left tibialis anterior muscle of the mice. To determine whether the co-administration of a plasmid DNA with poloxamers (synthetic triblock copolymers of poly(ethylene oxide) and poly(propylene oxide), also called Pluronics) in a mouse skeletal muscle can potentiate transgene expression in a mouse skeletal muscle, formulations of corresponding pDNA (10 µg) in 0.01% of SP1017 solution (mixture of Pluronics L61 and F127) were injected in the right tibialis anterior muscle of the same mice. Expression of luciferase reporter gene was monitored by non-invasive optical imaging using Kodak In Vivo Multispectral Imaging System (Carestream Health, Woodbridge, CT, USA) on day 4 and 7 post dosing. The images of the animals injected with gWIZ-Luc pDNA and pVAX1-Luc pDNA are presented in **Figure 16** and **Figure 17**, respectively.



**Figure 16.** gWIZ-Luc plasmid expression *in vivo* after a single i.m. injection of 10  $\mu$ g plasmid DNA in 50  $\mu$ l PBS. *In vivo* imaging was done on day 4 (**A** and **B**) and day 7 (**C** and **D**) after i.m. injection using the Kodak In Vivo Multispectral Imaging System. **E.** Quantitative analysis of the images for day 4 (D4) and 7 (D7) for each mouse.

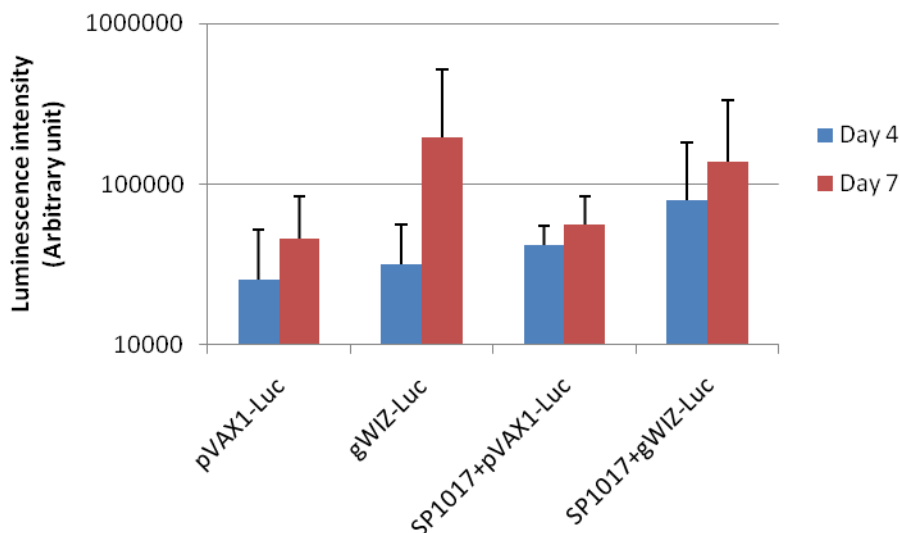


**Figure 17.** pVAX1-Luc plasmid expression *in vivo* after a single i.m. injection of 10  $\mu$ g plasmid DNA in 50  $\mu$ l PBS. *In vivo* imaging was done on day 4 (**A** and **B**) and day 7 (**C** and **D**) after i.m. injection using the Kodak In Vivo Multispectral Imaging System. **E.** Quantitative analysis of the images for day 4 (D4) and 7 (D7) for each mouse.

Expression of Luc transgene was observed in 3 out of 5 mice on day 4 after i.m. injection of naked gWIZ-Luc pDNA. The level of transgene expression was relatively high on day 7 post injection in all five animals studied (**Figure 16, A and C**). In the case of naked pVAX1-Luc pDNA, the Luc expression was detected only on days 4 and 7 in 2 animals out of 5 (**Figure 17, A and C**). The observed variations in levels of transgene expression between two groups could be related (1) to the technical errors upon pDNA injection or (2) to the differences in transcription efficiency among animals.

Importantly, an enhanced *in vivo* transgene expression was observed on day 4 and day 7 in the animals treated with the both gWIZ-Luc and pVAX1-Luc plasmid DNA formulated with 0.01% w/v SP1017 (**Figure 16 B,D,E**, and **Figure 17 B,D,E**). This is in good agreement with our previously reported data that co-administration of naked pDNA with Pluronic block copolymers increases the level of a plasmid DNA gene expression in skeletal muscle (9, 10). To compare the transgene expression of pVAX1-Luc pDNA (control plasmid) with gWIZ-Luc pDNA (reference plasmid) with and without 0.01% w/v SP1017, the average intensity of luminescence was plotted and presented in **Figure 18**. The average intensity of luminescence was measured within the region of interest (ROI) defined as the site of injection using Carestream Molecular Imaging software. It is seen the level of gWIZ-Luc pDNA expression was higher compared to pVAX1-Luc plasmid DNA. However, overall the collected data suggest that constructed pVAX1-Luc vector is also able to efficiently express the transgene *in vivo*.

On day 7 mice were sacrificed, muscles surrounding the site of injections were collected, snap frozen in liquid nitrogen and then stored at -80°C. The quantification of the Luc gene expression will be performed using Luciferase Assay Kit (Promega). It has been found that pVAX1-Luc vector also efficiently expressed the transgene as gWIZ-Luc in *in vivo*. pVAX1-Luc plasmid DNA formulated with 0.01% w/v SP1017 enhanced transgene expression in mice. Transgene expression of pVAX1-H5HA in the injected tibialis anterior muscles will be measured at mRNA levels by RT-PCR. Both these experiments are currently in progress.



**Figure 18.** Quantification of luminescence at the site of injection for the mice injected with 10  $\mu$ g of pVAX1-Luc and gWIZ-Luc plasmid DNA with/without 0.01% w/v SP1017 i.m. in Balb/c mice (5 per group). (A) *In vivo* imaging was done on day 4 (A and B) and day 7 (C and D) after i.m. injection.

#### KEY RESEARCH ACCOMPLISHMENTS

- Full length sequencing of the pMTH5 plasmid was performed and H5 gene sequence homology was obtained in comparison with the reference sequence provided on the NCBI GenBank database: DQ659326.1.
- Synthesis and characterization of highly parallel libraries of biodegradable polyanhydride nanoparticles based on two copolymer systems (CPH:SA and CPTEG:CPH) was successfully accomplished.
- Full length HA gene was successfully cloned into an insect cell secretion vector and stable cell lines were established. Purified protein is expected to be obtained within next six weeks.
- Cationic graft-copolymer based on polylysine (PLL-g-PEG) has been synthesized and characterized.
- The procedures for the synthesis of the cationic copolymers based on the polypeptides using ring-opening polymerization of  $\alpha$ -amino acid N-carboxyanhydrides were developed. Series of PEG-*block*-PLL were successfully synthesized and characterized.

- Series of cationic PEG-*block*-PLL copolymers containing mannosyl targeting groups were synthesized and characterized.
- DNA plasmids encoding H5N1 HA protein, pVAX1-H5HA, has been constructed, and sequenced. The expression of pVAX1-H5HA plasmid has been confirmed *in vitro* in HEK293T cells.
- DNA plasmid encoding reporter gene for luciferase (Luc), pVAX1-Luc, in place of influenza proteins has been successfully constructed and expressed.
- pVAX1-Luc vector was able to efficiently express the transgene *in vivo* post intramuscular injection as was determined by non-invasive animal optical imaging.
- Co-administration of pVAX1-Luc plasmid with Pluronics block copolymers (0.01% SP1017) increased the level of luciferase gene expression in skeletal muscle.

## REPORTABLE OUTCOMES

There are no reportable outcomes for the first year of the research program.

## CONCLUSIONS

- The procedures for the synthesis of the cationic copolymers based on the polypeptides using ring-opening polymerization of  $\alpha$ -amino acid N-carboxyanhydrides were developed. Series of PEG-*block*-PLL were successfully synthesized and characterized. The established synthetic procedures can be further expended to prepare various copolymers based on biodegradable and biocompatible polypeptides for the various biomedical applications.
- Synthesis and characterization of highly parallel libraries of biodegradable polyanhydride nanoparticles based on two copolymer systems (CPH:SA and CPTEG:CPH) was successfully accomplished. This achievement is very important for the following screening and identifying nanosphere formulations (i.e., chemistries and size) that exhibit exceptional cellular and tissue compatibility and have an ability to activate antigen presenting cells.
- A robust synthetic procedure for the incorporation of terminal reactive group into the block copolymers was developed. It will allow introducing various targeting moieties on

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